Mapping and dissection of bone morphogenetic protein signaling using genome-engineering tools

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Zusammenfassung der

KUMULATIVEN HABILITATIONSSCHRIFT

‘MAPPING AND DISSECTION OF BONE MORPHOGENETIC PROTEIN SIGNALING USING GENOME-ENGINEERING TOOLS’

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This Habilitation Thesis is based on the following original articles

Graf D, Timmons PM, Hitchins M, Episkopou V, Moore G, Ito T, Fujiyama A, Fisher AG, Merkenschlager M. 2001. Evolutionary conservation, developmental expression, and genomic mapping of mammalian Twisted gastrulation. Mamm Genome 12(7):554-560.

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The following book-chapter serves as introduction and general overview to the experimental approach taken in the work described:

Graf D, Economides AN. 2008. Dissection of BMP signaling using genome engineering tools. In: Vukicevic S, Sampath KT, editors. Bone Morphogenetic Proteins: From Local to Systemic Therapeutics. Basel/Switzerland: Birkhauser. p 115-139.

Introduction:

The development of embryonic tissues and structures, as well as their maintenance and repair in the adult, requires a very precise spatio-temporal regulation of the cellular events involved. Precursor cell recruitment, proliferation, differentiation, migration, and cell-death are all regulated by extracellular signaling networks, which serve to coordinate fate and function of individual cells within a given environment. The same networks that function throughout embryonic development also regulate homeostasis and repair of adult tissues. TGF-β/BMP/Smad, Wnt/frizzled, and Sonic hedgehog (Shh)/Patched signaling take central stage in all these processes. Understanding the regulation of any of these pathways, and therefore effects on the networks of which they are part of, is the challenge of ongoing research in biology. It is now clear that any secreted ligand-driven cellular response is not only regulated by sophisticated intracellular positive or negative feedback mechanisms, but that the availability of the ligand outside of the cell is also very strictly regulated. This is exemplified in the family of Bone Morphogenetic Proteins (BMPs).

BMPs are a large subgroup of phylogenetically conserved molecules belonging to the TGF-ß superfamily. BMPs are multifunctional proteins and exert a wide range of biological functions in a variety of cell types, including epithelial and mesenchymal cells. Although BMPs were originally identified as the molecules that induce bone and cartilage formation at ectopic sites in rats - hence their name Bone Morphogenetic Proteins - they regulate many aspects of embryonic development, in particular cell differentiation, growth/apoptosis and, thus, have pivotal roles in morphogenesis of most tissues and organs. The activity of Bone Morphogenetic Proteins is regulated in the extracellular space by a complex network of BMP binding proteins. BMP antagonists, like Gremlin, Noggin, Chordin and its partner Twisted Gastrulation, bind to BMPs and shape the BMP activity gradient by directly regulating the ability of BMPs to bind to their receptors. To understand BMP function, functional dissection of this extracellular network is required, and this involves both detailed knowledge of BMP/BMP antagonist expression as well as adequate tools to allow genetic manipulation such as gene ablation *in vivo*.

This cumulative habilitation encompasses four studies that illustrate various aspects of a concerted effort to analyze the BMP signaling network in embryonic and adult tissues. It describes the cloning of the mammalian orthologue of *Drosophila* Twisted Gastrulation, its role in regulating T-cell development in the thymus, a general strategy for engineering conditional alleles exemplified on *Bmp7*, and how LacZ-mediated gene reporting, again exempliefied on *Bmp7*, can be used to map expression and to predict its function in the developing embryo. In summary, the studies provide an example on how the combination of gene identification, high resolution gene expression analysis, high precision genome engineering, and simplified cellular model systems allow dissection of a complex signaling network *in vivo* such as BMP signaling (Graf and Economides, 2008).

Discussion

Twisted Gastrulation (tsg) was identified in a screen for novel genes expressed during lymphocyte development and activation. The human, mouse, zebrafish, and *Xenopus Tsg* homologs described in the first publication (Graf et al., 2001) encode vertebrate proteins that are closely related (40% identical and > 50% similar) to the *Drosophila tsg* gene product. Shared features include all 24 cysteine residues and a hydrophobic signal sequence, indicating that Tsg has been conserved as a secreted protein of defined secondary structure over large evolutionary distances. In contrast to *Drosophila,* where *tsg* expression is limited to the blastoderm and early germband

extension, mammalian *Tsg* is expressed throughout embryonic development and persists in the adult. The evolutionary conservation of the Tsg protein reflects the conserved interaction with BMP, though the more persistent expression may indicate the acquisition of additional Tsg functions in mammals. Embryonic patterning depends on a gradient of BMP activity, and perturbations can result in defective development. Mice lacking *Tsg* display an array of craniofacial malformations, which are in part caused by increased apoptosis in the 1st branchial arch (MacKenzie et al., 2009). Similarly to the loss of midline in the mice, the lack of *tsg* in *Drosophila* causes dorsal midline defects at the amnioserosa. In the absence of tsg, cells at the amnioserosa are initially specified but subsequently cannot be maintained a finding reminiscent of the degeneration of forebrain and midline structures induced by BMP overexposure in chick embryos. Of interest to note is that *TSG* is located on human Chromosome 18p11.3 mapping close to the Holoprosencephaly (HPE)-4 locus, though no studies have been published linking genetic alterations in *Tsg* to HPE (Graf et al., 2001).

The second publication (Graf et al., 2002) was based on the observation that Tsg is expressed in immature thymocytes in a developmentally regulated fashion. Pre-T cell receptor (TCR) and TCR signaling resulted in increased Tsg expression at two developmental checkpoints, the double negative (DN) to double positive (DP) and the DP to single positive (SP) transition. Using embryonic thymi it was shown that DN thymocytes were highly susceptible to BMP4, which acted directly and without relay by another cell type to reduce DN thymocyte proliferation and progression to the DP stage *in vitro*. Bmp2, Bmp4, as well as the tsg-partner Chordin were all exclusively expressed by thymic stroma cells. Thus a model was proposed whereby BMP2/BMP4 signals derived from the thymic stroma control maturation of thymocyte precursors by reducing both their rate of proliferation and differentiation. When individual precursors receive a pre TCR-dependent competence signal to proceed with their maturation program, they up-regulate Twisted Gastrulation, which in synergy with stroma-derived Chordin alleviates this BMP effect. These effects were specific to BMP2/BMP4 as BMP7 had no effect on thymocyte maturation. The finding of developmentally regulated *Tsg* expression in the thymus extends the concept that cells within a morphogenetic field not only read and respond to the local morphogen concentration, but also can be instrumental in shaping the morphogen gradient. It suggests that cells can temporarily withdraw from signaling molecules affecting their differentiation via the increased expression of a secreted modifier at specific developmental control points. This concept has major implications on our understanding on how BMP signaling works and it will be important in other biological settings such as craniofacial development or tissue repair.

The data in the thymus were collected using a combination of *in situ* expression profiling, expression studies on isolated cells, and *ex vivo* organ cultures. To obtain proof that this BMP/BMP antagonist interaction operates in the proposed manner *in vivo* and to facilitate functional dissection of this network, detailed knowledge of BMP/BMP antagonist expression *in vivo* as well as tools for their gene ablation were required. The third report (Zouvelou et al., 2009b) describes the generation of a conditional allele for *Bmp7* through gene targeting by homologous recombination. Gene targeting, by definition, involves introduction of changes into the genome. Any such process potentially introduces changes that may affect the expression of nearby genes. Many *Bmp* genes are located in apparent 'gene-deserts', meaning in relative isolation from neighboring genes. This is likely

due to the presence of long-range regulatory elements that fine tune *Bmp* gene expression. Indeed, studies on Bmp4 and Bmp5 have shown that tissue and cell-specific enhancers are located several hundred kb up- and downstream of the actual exons. These enhancers are often evolutionary conserved and as we have shown elsewhere for *Bmp4*, the serendipitous introduction of as little as 50bps in one of these evolutionary conserved elements can result in discernable phenotypic changes (Graf and Economides, 2008). For this reason, our strategy employed Bacterial Homologous Recombination (BHR) to generate Bacterial Artificial Chromosome (BAC)-based targeting vectors. BHR, also commonly referred to as 'recombineering', revolutionized genome engineering as it enabled the precise assembly of DNA sequences, independent of the presence of restriction sites and the size of the DNA molecule to be modified. As this technology allows genome manipulation with base-pair precision, it enabled to consider locus structure and to avoid the generation of such hypomorphic alleles. The classical two-loxP site strategy for the generation of conditional alleles was adapted to Bacterial Homologous Recombination to create a Bacterial Artificial Chromosome-based vector for direct targeting in mouse embryonic stem cells. Functional analysis showed that *in vivo*, the conditional-null Bmp7flx/flx mice are phenotypically wild type, whereas post Cre-mediated recombination, the resulting Bmp7Δ/Δ mice are phenotypically null. Thus, this study validated the usefulness of the Bmp7flx/flx mouse, which in turn should empower in vivo studies aimed at elucidating the roles of Bmp7 in postnatal development, homeostasis and disease.

The fourth study (Zouvelou et al., 2009a) describes the expression of BMP7 throughout embryonic craniofacial development using a lacZ reporter allele and correlates expression to phenotypic changes observed in homozygous, germline deleted, conditional BMP7-deficient mice. The study covered the whole spectrum of orofacial development and was not restricted to either individual structures or single time points. BMP7 expression was detected in both mesenchymal and epithelial structures of the developing head, whereas an epithelial expression pattern was observed in all ectodermal appendages of the orofacial region. In line with the expression data, severe craniofacial malformations were observed in Bmp7 deficient (Bmp7Δ/Δ) embryos including cleft palate. BMP7 expression in the developing teeth was dynamic and often locally restricted. In Bmp7Δ/Δ embryos the maxillary incisors were often missing, the mandibular incisors were deformed and hypoplastic, and in cases only one mandibular incisor developed. The maxillary and mandibular first molars were developmentally delayed and sometimes misplaced or missing. A more general involvement of BMP7 in the development of ectodermal appendages and thereby epithelial–mesenchymal interactions was indicated by the strong expression of BMP7 in developing hair follicles and salivary glands, and the concomitant morphological deformation of these two structures. This report established novel non-redundant roles for BMP7 in the development of the orofacial region and places BMP7 as a central player in the development of ectodermal appendages and other structures involving mesenchymal–epithelial interactions such as the palate. In addition, the present findings suggest that BMP7 might regulate stem and/or progenitor cells in dental and non-dental tissues. Of particular interest is BMP7 expression in cells reminiscent of delaminating neural crest, and the almost complete loss of some neural crest derived structures in Bmp7-deficient embryos.

In summary, this cumulative habilitation shows how the combination of biological systems, high-resolution expression analysis, and high precision genome engineering empowers functional studies on BMP biology *in vivo*.

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